# Allele-specific hybridization using streptavidin-coated magnetic beads for species identification, S genotyping, and SNP analysis in plants



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# Allele-specific hybridization using streptavidin-coated magnetic beads for species identification, *S* genotyping, and SNP analysis in plants

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## Abstract

**Dot-blot hybridization has been successfully used for construction of linkage maps of SNP markers, QTL analysis, marker-assisted selection, and identification of species and cultivars, but this method is time-consuming, even for a small number of plant samples. Instead of the nylon membrane used for dot-blot hybridization, we used streptavidin-coated magnetic beads to immobilize PCR products and hybridized allele-specific oligonucleotide probes and a digoxigenin-labeled oligonucleotide, which was hybridized with the allele-specific oligonucleotide probes. After amplification of plant DNA by PCR with biotinylated oligonucleotides, oligonucleotide probes having species-specific or allele-specific sequences, the digoxigenin-labeled oligonucleotide, and streptavidin-coated magnetic beads were mixed under temperature suitable for each probe. Species-specific internal transcribed spacer 1 (ITS1) sequences and allele-specific sequences of hypervariable region I of *S*-locus receptor kinase (*SRK*) specifically detected ITS1 sequences and *SRK* alleles in *Brassica* species, respectively. SNPs were also successfully analyzed by using allele-specific oligonucleotide probes and competitive oligonucleotides. In SNP analysis, PCR products were indirectly captured by magnetic beads. SNP alleles of eight cultivars each in *Brassica rapa* and *Raphanus sativus* were analyzed using streptavidin-coated magnetic beads, and genotyping results corresponded well with those of dot-blot-SNP analysis. Although allele-specific hybridization using streptavidin-coated magnetic beads is somewhat costly, it is rapider and easier than dot-blot hybridization. This method is suitable for analysis of a small number of plant samples with a large number of DNA markers.**

**Keywords: *Brassica*, DNA markers, dot-blot hybridization, ITS1, self-incompatibility, *SRK***

## Introduction

DNA polymorphism analysis is indispensable for developing DNA markers for mapping and map-based cloning of genes in plant genetic study and marker-assisted selection in plant breeding. Simple sequence repeat (SSR) markers are commonly used for these purposes by many plant geneticists and breeders (Morgante and Olivieri 1993; Thomas and Scott 1993; Wu and Tanksley 1993). In many cases, however, analysis of SSR markers requires electrophoresis of long polyacrylamide gels. Sample application, electrophoresis, and staining of DNA are time-consuming and labor-intensive. Furthermore, this method can analyze only repeat sequences, and is thus not applicable for analysis of nucleotide sequence polymorphism of particular genes. Single nucleotide polymorphism (SNP) is the most abundant DNA polymorphism in genomes. A large number of genetic differences between alleles of genes are due to SNPs. Therefore, SNP analysis enables production of high-density linkage maps of DNA markers, map-based cloning of genes using closely linked DNA markers, and selection of plants with desirable alleles.

Many methods for SNP analysis have been reported, but most such methods are costly (Shen et al. 2005) or labor-intensive (Orita et al. 1989; Michaels and Amasino 1998; Till et al. 2004). Although allele-specific PCR is simple, rapid, and cost-effective (Latorra et al. 2003; Zhou et al. 2004), genotyping data by this method are sometimes less reliable, because the genotyping depends on the success or failure of DNA amplification by PCR. Monitoring of allele-specific PCR using real-time PCR is a means of solving this problem. Analysis of melting temperature of double strand DNA is also a useful method for SNP analysis (Wittwer et al. 2003). However, these methods require special equipment. Therefore, we have developed the dot-blot-SNP method (Shirasawa et al. 2006; Shiokai et al. 2010). Genotyping data obtained by this method are highly reliable and suitable for analysis of a large number of plants (Shirasawa et al. 2006). This method has been successfully used for construction of linkage maps (Li et al. 2009, 2011), QTL analyses (Udagawa et al. 2010; Sato et al. 2011; Shirasawa et al. 2012), marker-assisted selection (Shirasawa et al. 2008), and identification of cultivars (Sato et al. 2010). However, analysis of a small number of samples requires time as long as that for a large number of samples. A simple, rapid method for reliable SNP genotyping is still required.

It is sometimes difficult to identify plant species. For example, *Brassica juncea* is similar to *Brassica rapa* and *Brassica napus*. Gloss and shape of leaves are different between them, but such traits vary within a species. The only reliable way to identify these species has been chromosome count. Differences of nucleotide sequences between different species in many genes have been published in DNA databases. Recently, we have reported the availability of dot-blot hybridization of rDNA internal transcribed spacer 1 (ITS1) sequences for identification of species in the tribe Brassiceae and designed species-specific probes (Tonosaki and Nishio 2010). In the breeding or fruit production of self-incompatible crops, identification of *S* haplotypes is required. PCR-RFLP (CAPS) analyses of the *S-locus glycoprotein* (*SLG*) gene in Brassicaceae (Nishio et al. 1994; Sakamoto et al. 2000) and the *S-RNase* gene in Rosaceae (Ishimizu et al. 1999) have been widely used for these purposes. With these methods, *S* haplotypes can be distinguished from each other, but identification of *S* haplotypes is difficult (Sakamoto and Nishio 2001). Methods for *S* haplotype identification of

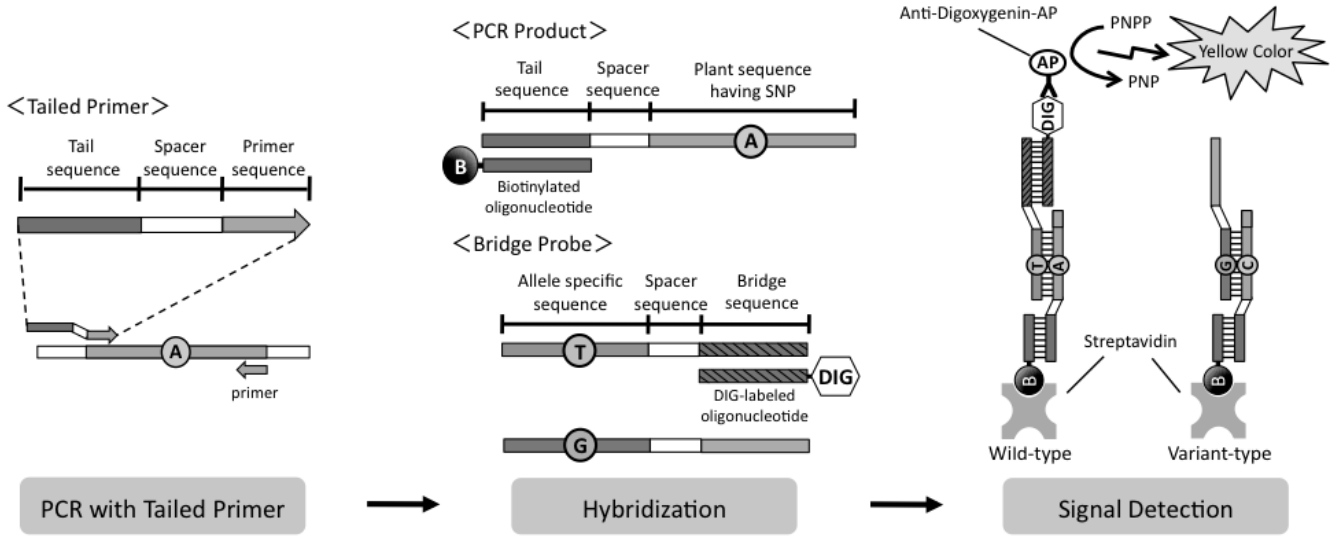


**Fig. 1.** Scheme of magnetic bead hybridization for SNP analysis
In SNP analysis, PCR products were indirectly captured by streptavidin-coated magnetic beads using a tail sequences. In analyses of ITS1 and *SRK*, biotinylated primers were used in PCR for direct capture of PCR products by streptavidin-coated magnetic beads.

Brassicaceae and Rosaceae using dot-blot hybridization have been developed (Fujimoto and Nishio 2003; Kitashiba et al. 2008; Oikawa et al. 2011). However, analysis with these methods is also time-consuming.

By capturing the PCR products with biotin-streptavidin binding, allele-specific hybridization can be detected. PCR-ELISA, which is an enzyme-linked immunosorbent assay of PCR products using the biotin-streptavidin system (Landgraf et al. 1991; Gibellini et al. 1993), is widely used for diagnosis of viral, bacterial, and fungal diseases in humans (Borrow et al. 1997; Loffler et al. 1998; Munch et al. 2001) and plants (Bonants et al. 1997; Olmos et al. 1997; Bailey et al. 2002; Tamminen et al. 2004). However, this method has seldom been used in genetic studies of plants (Zuniga et al. 2008). Streptavidin-coated magnetic beads can be used instead of a streptavidin-coated microtiter plate for PCR-ELISA and a nylon membrane for dot-blot hybridization. In the present study, we examined allele-specific hybridization using streptavidin-coated magnetic beads, herein termed "magnetic bead hybridization", an outline of which is shown in Fig. 1, in analyses of species-specific ITS1 sequences, allele-specific sequences of *S-receptor kinase* (*SRK*), and SNPs of genes using Brassicaceae crops, and compared it with dot-blot hybridization.

## Materials and Methods

Plant materials and preparation of genomic DNA

Thirteen species of the tribe Brassiceae, i.e., seven species in *Brassica*, one in *Diplotaxis*, one in *Eruca*, one in *Moricandia*, two in *Sinapis*, and one in *Raphanus,* all maintained in the *Brassica* Seed Bank of Tohoku University listed in Table 1, were used as plant materials for examining magnetic bead hybridization. Homozygous plants for seven *S* haplotypes in *Brassica rapa* and six *S* haplotypes in *Brassica oleracea* (Oikawa et al. 2011) were used as materials for analysis by magnetic bead hybridization with *S* haplotype-specific oligonucleotide probes designed from published *S*-receptor kinase (*SRK*) sequences (Sato et al. 2002). A doubled haploid 'P11' of a Komatsuna cultivar 'Osome' and an inbred line 'C634' of an Indian oilseed cultivar 'Yellow sarson' in *B. rapa* were used for analysis of SNPs in *BrFLC1, BrFLC2, BrGL1*, and *MLPK*. Using magnetic bead hybridization and dot-blot SNP, eight cultivars each of *B. rapa* and *Raphanus sativus* were genotyped with eight SNP markers reported in our previous studies (Li et al. 2010, 2011; Udagawa et al. 2010). Genomic DNAs were prepared from 10 mg of leaf tissues according to the method of Edwards et al. (1991). Seed DNA was extracted from each grain using the NaI method (Sakamoto et al. 2000).

Preparation of oligonucleotide primers and probes

The oligonucleotide primers and probes used in this study are listed in Supplementary Table S1, S2, and S3. For amplification of the internal transcribed spacer 1 (ITS1) region in ribosomal DNA, the specific primer ITS1-18S (5'-CGTAACAAGGTTTCCGTAGG-3'; Venora et al. 2000) and a newly designed biotinylated ITS1-5.8S (5'-CGTTCTTCATCGATGCGAGA-3') were used. The species-specific oligonucleotide probes reported in our previous study (Tonosaki and Nishio 2010) and newly designed oligonucleotide probes were used (Supplementary Table S1). For amplification of hypervariable region I (HV1) of *SRK*, a primer HV1-F (5'-TTGCTTCCAGAGATGAAACTGGG-3') and a biotinylated primer HV1-R (5'-CGGATTCCATTCCATGGACC-3') were designed by comparing the HV1 region of *SRK* sequences (Sato et al. 2002) between different *S* haplotypes in *B. rapa* to select 17 - 25 bp haplotype-specific sequences. Species-specific oligonucleotide probes (Supplementary Table S1) and *S* haplotype-specific oligonucleotide probes (Supplementary Table S2) were prepared as bridge probes for indirect hybridization with a digoxygenin-labeled oligonucleotide probe according to Shiokai et al. (2010). These bridge probes were composed of a probe sequence for detection, a 6-bp spacer sequence of TATATT, and a BrSCR27 sequence (5'-TACATTCGCAATTGAGGCTTCGT-3') complementary to

the sequence of the digoxygenin-labeled oligonucleotide probe.

For SNP analysis, four sequences of *BrFLC1*, *BrFLC2*, *BrGL1* (Li et al. 2010), and *BrMLPK* (Murase et al. 2004) in *B. rapa* were used for designing primers to amplify fragments containing SNPs. Primers at one side were prepared as tailed primers for indirect capture by streptavidin. These tailed primers were composed of a BrSCR12 sequence (5'-TGCGAAAACACATACAAACGTCTGA-3') complementary to the sequence of biotinylated oligonucleotide, a 6 bp spacer sequence of TATATT, and a primer sequence to amplify DNA fragments containing SNPs. The bridge probe for detection of SNPs of one allele was the same as that for analysis of ITS1 and *SRK*, but another bridge probe having sequences of SNPs, a 6-bp spacer sequence of TATATT, and a BrSCR52 bridge sequence was used for detection of the other allele (Supplementary Table S3).

Dot-blot hybridization

DNA fragments were amplified by PCR using the primers listed in Supplementary Table S3. A PCR product was mixed with an equal volume of a denaturation solution containing 0.4 N NaOH and 10 mM EDTA and dot-blotted onto a nylon membrane (Hybond-N, GE Healthcare UK) by Multi-pin Blotter (ATTO, Japan). After UV crosslinking using Gene Linker UV Chamber (Bio-Rad Laboratories, USA), the membrane was hybridized with a probe for 2 h or overnight at the temperature shown in Supplementary Table 3. After hybridization, the membrane was washed twice by 2 x SSC containing 0.1% SDS at room temperature for 5 min, and then again under the same temperature for hybridization with the solution listed in Supplementary Table S3. The hybridized digoxygenin-labeled probe was detected by an anti-digoxygenin IgG Fab fragment conjugated with alkaline phosphatase (Roche, Germany) followed by chemiluminescent reaction of CSPD (Roche). Chemiluminescence was exposed to X-ray film (Fuji, Japan). Signal densities were measured by ImageJ (http://rsbweb.nih.gov/ij/).

Allele-specific hybridization using streptavidin-coated magnetic beads (magnetic bead hybridization)

Before use, the streptavidin-coated magnetic beads were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) three times. Streptavidin-coated beads (50 µg, Streptavidine Magnetic Particles, Roche, Germany) were added to a PCR tube containing 5 µl biotinylated PCR product and 100 µl PBST, and incubated at 37˚C for 30 minutes. After the addition of 50 µl denaturation solution (0.5 N NaOH) to remove a complementary strand of DNA and washing twice with PBST, captured DNA was incubated in 200 µl of hybridization buffer (5 x SSC with 0.3% Tween-20) containing an allele-specific oligonucleotide probe (Supplementary Table S1, S2, and S3) and DIG-labeled oligonucleotide probe having the complementary sequence of the bridge sequence in the allele-specific oligonucleotide probe for 1 hour or overnight at the temperature shown in Supplementary Table S1, S2, and S3. After hybridization with a probe, DNA was washed with PBST at room temperature twice. The hybridized digoxygenin-labeled probe was combined with an anti-digoxygenin IgG Fab fragment conjugated with alkaline phosphatase (Roche, Germany) in PBST for 30 minutes at room temperature, washed with PBST at room temperature twice, and detected by coloring reaction with p-nitrophenyl phosphate (Thermo; USA). Color density was measured at 405 nm by iMark Microplate Reader (BIO RAD, USA) and analyzed by Microplate Manager 6.0 (BIO RAD, USA). In the case using the tailed primer, PCR products amplified by tailed primers were hybridized with biotinylated oligonucleotide and probes in hybridization buffer (5 x SSC with 0.3% Tween-20) together with streptavidin-coated beads. After hybridization, the procedure described above was used for detection of hybridized DNA.

## Results

Magnetic bead hybridization of ITS1 sequences

For identification of species, we newly designed nine probes having species-specific sequences in the ITS1 region. One species-specific probe, i.e., *Diplotaxis muralis*, had the same sequences as that reported by Tonosaki and Nishio (2010). A pair consisting of a biotinylated primer and an unlabeled primer was used for amplification of the ITS1 region by PCR. Using the ten species-specific probes, 13 species including amphidiploid species, i.e., *Brassica napus, Brassica juncea*, and *Brassica carinata,* were analyzed twice. Averages of relative absorbance values, which are absorbance values divided by the values of full matching between the probe sequences and amplified sequences, are shown in Table 1. Most of the species analyzed by species-specific probes for different species showed relative absorbance values lower than 0.2.

A species-specific probe for *B. rapa*, which has the A genome, detected PCR products of *B. napus* having the AC genome and *B. juncea* having the AB genome with relative absorbance values of more than 0.8. A species-specific probe for *Brassica nigra*, which has the B genome, detected PCR products of *B. carinata* having the BC genome and *B. juncea* with relative absorbance values more than 0.8. Relative absorbance values of *B. napus* and *B. carinata* analyzed by a species-specific probe for *B. oleracea* having the C genome were also more than 0.80. These results indicate that amphidiploid species can be identified by the probes for monogenome species, the genome of which constitutes the genome of the amphidiploid species.

Magnetic bead hybridization of SRK sequences

Seven oligonucleotide probes specific to *SRK* alleles in *S-12, S-25, S-27, S-36, S-41*, *S-52,* and *S-54* of *B. rapa* were designed using hypervariable region I (HV1) sequences (Sato et al. 2002). All the probes detected allele-specific signals with low background signals except for an allele-specific probe of *S-52*, which showed a relative absorbance value of 0.38 in analysis of PCR products from an *S-27* homozygote (Table 2).

*B. rapa* is closely related to *B. oleracea*, and these two species share the same *S* haplotypes having the same recognition specificities with highly similar nucleotide sequences of *SRK* and *SP11/SCR* (Sato et al. 2003). Such a combination of *S* haplotypes in different species is termed "interspecific pair." Interspecific pairs of *S-12, S-25, S-27, S-36, S-41*, and *S-54* in *B. rapa* are *S-51, S-14, S-8, S-24, S-64*, and *S-28* of *B. oleracea*, respectively. PCR products from *S-51, S-14, S-8, S-24, S-64*, and *S-28* in *B. oleracea* were also detected specifically by the allele-specific oligonucleotide probes of the interspecific pairs in *B. rapa*.

Table 1. Detection of species-specific signals in 14 species in Brassiceae by magnetic bead hybridization using species-specific ITS1 probes

| Species names and accession numbers | ITS1 probe names | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | BrITS 1 | BoITS 1 | BniITS 1 | BtITS 1 | DmITS 1 | EsITS 1 | MaITS 1 | SalITS 1 | SarITS 1 | RsITS 1 |
| *Brassica rapa* C-101 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.06 | 0.00 |
| *Brassica oleracea* O-121 | 0.07 | 1.00 | 0.06 | 0.00 | 0.00 | 0.00 | 0.02 | 0.07 | 0.06 | 0.00 |
| *Brassica nigra* Ni-109 | 0.05 | 0.06 | 1.00 | 0.07 | 0.00 | 0.00 | 0.00 | 0.07 | 0.06 | 0.06 |
| *Brassica napus* N-102 | 0.92 | 0.89 | 0.09 | 0.00 | 0.00 | 0.00 | 0.01 | 0.09 | 0.06 | 0.01 |
| *Brassica juncea* J-103 | 0.88 | 0.07 | 0.98 | 0.02 | 0.00 | 0.00 | 0.01 | 0.09 | 0.05 | 0.01 |
| *Brassica carinata* Ca-104 | 0.18 | 0.80 | 0.83 | 0.05 | 0.00 | 0.00 | 0.00 | 0.08 | 0.07 | 0.00 |
| *Brassica tournefortii* T-134 | 0.02 | 0.00 | 0.01 | 1.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.06 | 0.00 |
| *Diplotaxis muralis* DIP-MUR-3 | 0.03 | 0.00 | 0.00 | 0.00 | 1.00 | 0.00 | 0.03 | 0.07 | 0.06 | 0.00 |
| *Eruca sativa* ERU-SAT-1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 0.00 | 0.09 | 0.06 | 0.00 |
| *Moricandia arvensis* MOR-ARV-4 | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 1.00 | 0.08 | 0.06 | 0.00 |
| *Sinapis alba* SIN-SLB-1 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 0.06 | 0.03 |
| *Sinapis arvensis* SIN-ARV-1 | 0.03 | 0.00 | 0.11 | 0.00 | 0.00 | 0.00 | 0.00 | 0.09 | 1.00 | 0.01 |
| *Raphanus sativus* cv. Shogoin | 0.03 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.06 | 1.00 |

Relative absorbance values (absorbance of 405 nm) after 1 hour to the full matching (1.00) are shown. Values higher than 0.8 are regarded as positive, while those lower than 0.2 are regarded as negative.

Table 2. Detection of *S* haplotype-specific signals in sevens haplotypes of *B. rapa* and six haplotypes of *B. oleracea* by magnetic bead hybridization using allele-specific *SRK* probes

| *S* haplotypes | *S RK* probe names | | | | | | |
|---|---|---|---|---|---|---|---|
| | BrS-12HV 1 | BrS-25HV 1 | BrS-27HV 1 | BrS-36HV 1 | BrS-41HV 1 | BrS-52HV 1 | BrS-54HV 1 |
| *Brassica rapa* | | | | | | | |
| *BrS-12* | 1.00 | 0.00 | 0.04 | 0.14 | 0.05 | 0.07 | 0.07 |
| *BrS-25* | 0.09 | 1.00 | 0.05 | 0.15 | 0.06 | 0.08 | 0.09 |
| *BrS-27* | 0.10 | 0.00 | 1.00 | 0.14 | 0.05 | 0.38 ±0.27 | 0.10 |
| *BrS-36* | 0.10 | 0.00 | 0.05 | 1.00 | 0.06 | 0.08 | 0.09 |
| *BrS-41* | 0.13 | 0.00 | 0.05 | 0.15 | 1.00 | 0.09 | 0.09 |
| *BrS-52* | 0.09 | 0.00 | 0.06 | 0.15 | 0.05 | 1.00 | 0.08 |
| *BrS-54* | 0.11 | 0.00 | 0.06 | 0.17 | 0.06 | 0.09 | 1.00 |
| *Brassica oleracea* | | | | | | | |
| *BoS-8* | 0.10 | 0.00 | 0.62 ±0.27 | 0.15 | 0.06 | 0.08 | 0.09 |
| *BoS-14* | 0.08 | 0.97 | 0.05 | 0.15 | 0.06 | 0.08 | 0.09 |
| *BoS-24* | 0.10 | 0.00 | 0.05 | 0.78 ±0.18 | 0.06 | 0.08 | 0.09 |
| *BoS-28* | 0.09 | 0.00 | 0.05 | 0.15 | 0.06 | 0.08 | 0.76 ±0.16 |
| *BoS-51* | 0.80 ±0.18 | 0.00 | 0.05 | 0.15 | 0.06 | 0.08 | 0.08 |
| *BoS-64* | 0.11 | 0.00 | 0.06 | 0.16 | 0.78 ±0.19 | 0.08 | 0.08 |

Relative absorbance values after 1 hour to the full matching (1.00) are shown. Values higher than 0.8 are regarded as positive, while those lower than 0.2 are regarded as negative. For the combinations with relative absorbance values between 0.2 and 0.8, analysis was repeated three times and SE was calculated.

Magnetic bead hybridization for SNP analysis

Magnetic bead hybridization was compared with dot-blot hybridization using the four SNP markers of *BrFLC1, BrFLC2, BrGL1,* and *BrMLPK*. PCR products were successfully detected in an allele-specific manner by both dot-blot analysis and magnetic bead hybridization. Intensities of non-specific signals were generally lower in magnetic bead hybridization than those in dot-blot analysis (Fig. 2).

Genotyping results of eight cultivars each of *B. rapa* and *R. sativus* with magnetic bead hybridization are shown in Supplementary Table S4 and S5, respectively. Combinations of cultivars and SNP markers that showed positive signals by dot-blot-SNP analysis had relative absorbance values higher than 0.5 in magnetic bead hybridization with five exceptions, i.e., 'Nozaki' with BrRSCL6068-Chiifu probe (Supplementary Table S4), 'Aokubi' with RsCL1111-Saya probe, 'Sakurajima' with RsCL0976-Aokubi probe and RsCL1033-Aokubi probe, and 'Karaine' with RsCL1179-Saya probe (Supplementary Table S5). In these exceptions, dot-blot-SNP analysis showed positive signals, while relative absorbance values in magnetic bead hybridization were less than 0.5. Nucleotide sequence analysis revealed that there are other SNPs within the probe sequences in these combinations of cultivars and markers. For example, BrRSCL6068-Chiifu probe had a sequence of 5'-AAGGAAGCAAGAAGAAA-3', while 'Nozaki' had a sequence of 5'-AAGCAAGCAAGAAGAAA-3'.

No signals were detected in 'Comet' by both allele-specific probes of RsCL1111, and in 'Shogoin', 'Sakurajima', 'Moriguchi', 'Karaine', and 'Comet' by both allele-specific probes of

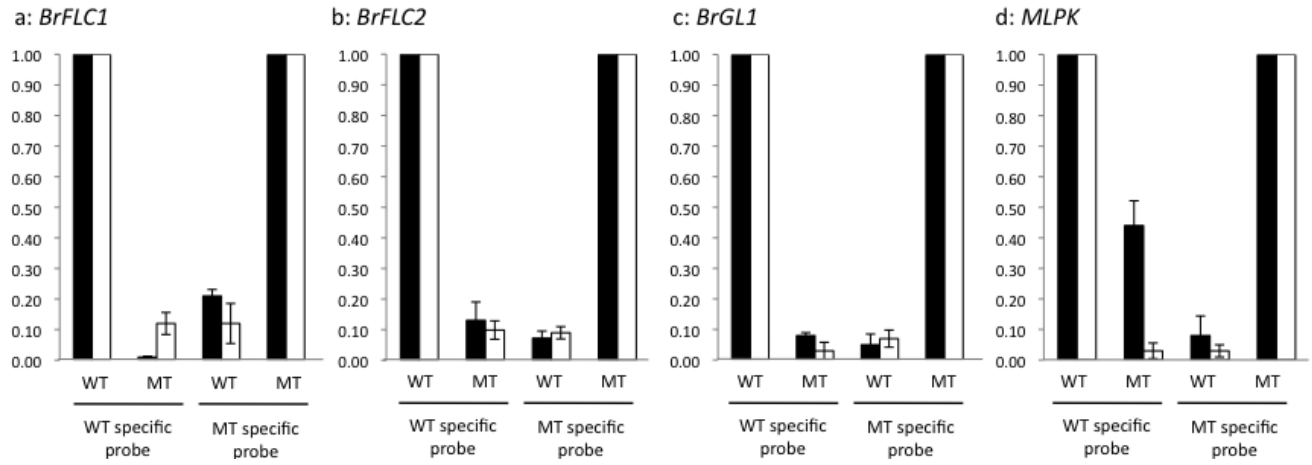


Fig. 2. Comparison of nonspecific signal intensities between magnetic bead hybridization and dot-blot hybridization in SNP analysis
Using four SNP markers, plants homozygous for wild type (WT) and mutant type (MT) were analyzed. Black bars and white bars are relative absorbance values, in which absorbance at 405 nm of analysis of full-matched sequences was set to 1.00, of dot-blot hybridization and magnetic bead hybridization, respectively. SE of five analyses are shown.

RsCL1179. Nucleotide sequence analysis of PCR products revealed that these cultivars have a different nucleotide sequence from those of both alleles used for these SNP markers. 'Comet' had a sequence of 5'-TCAATAGTCCTGACA-3' at the position used for probes of RsCL1111 (5'-TCAAGAGTCTGAACA-3'). 'Sakurajima', 'Moriguchi', and 'Karaine' had 5'-ATGAAAATCTTCTA-3', 'Shogoin' had 5'-ATGAAACAATTTCTA-3', and 'Comet' had 5'-ATGAAACAATCTTCTA-3' at the position used for probes of RsCL1179.

## Discussion

Magnetic bead hybridization with ten species-specific oligonucleotide probes of ITS1 sequences was effective for detection of PCR products of species, sequences of which were used for designing the probes, namely, full matching. Tonosaki and Nishio (2010) have reported dot-blot hybridization of genomic DNA using ITS1 sequences as probes to be useful for identification of species. However, their method requires a large amount of purified genomic DNA. Magnetic bead hybridization was shown to be much easier than dot-blot hybridization and to be useful for identification of species in the tribe Brassiceae.

Magnetic bead hybridization with seven allele-specific oligonucleotide probes of *SRK* enabled allele-specific detection of *SRK* in *B. rapa*. Furthermore, *SRK* alleles in *B. oleracea* having the same recognition specificities as those in *B. rapa* were detectable with the allele-specific probes for *B. rapa*. In an *S-27* homozygote of *B. rapa*, relatively high signals were detected by a BrS-52HV1 probe. Although the *SRK* allele of *S-27* has low nucleotide sequence identity with the sequence of the BrS-52HV1 probe, i.e., 74%, an *SLG* allele of *S-27* has relatively high homology with the BrS-52HV1 probe, i.e., 87% (Kusaba et al. 1997). Since the primer pair used in this analysis can also amplify *SLG* alleles, relatively high background signals due to high homology between *SRK* and *SLG* may be detected. For designing allele-specific probes for *SRK* alleles, sequences of *SLG* alleles in different *S* haplotypes should be considered.

Oikawa et al. (2011) have found that dot-blot hybridization of *SP11/SCR* alleles is useful not only for identification of *S* haplotypes but also for screening of particular *S* haplotypes in *Brassica* population. In their method for *S* haplotype identification, multiplex PCR with many primer pairs for different *SP11/SCR* alleles is used for digoxigenin labeling of PCR products. Since there are more than 50 *S* haplotypes in a species of *Brassica* (Ockendon 2000; Nou et al. 1993), their method eventually requires more than 50 primer pairs for multiplex PCR. In the present method, the hypervariable region I of *SRK* was used for designing allele-specific probes, and relatively conserved regions were used for designing primers for amplification of many *SRK* alleles. PCR using a single pair of primers is easier and more reliable than multiplex PCR. Although further analyses using more allele-specific probes are required for establishment of magnetic bead hybridization using *SRK* alleles for *S* haplotype identification, this method is expected to be useful as an *S* haplotype identification method.

SNPs were also successfully analyzed by magnetic bead hybridization. In analyses of SNP markers, to reduce the cost of synthesizing primers for many SNP markers, oligonucleotides having a tail sequence were used as primers for amplification of plant DNA instead of biotinylated oligonucleotides. Non-specific signals were lower in analyses by magnetic bead hybridization than those by dot-blot hybridization. Since quantitative data are obtained in magnetic bead hybridization, genotyping data of magnetic bead hybridization are more reliable than those of dot-blot hybridization. A method for SNP analysis using streptavidin-coated beads has recently been reported by Liu et al. (2011). They used a primer extension method with FITC-labeled dideoxynucleotides and anti-FITC antibody. This method requires a biotinylated primer for every SNP marker. PCR-ELISA has been used for SNP analysis (Knight et al. 1999). The principle of analysis of magnetic bead hybridization is the same as that of PCR-ELISA, but a streptavidin-coated multititre plate used for PCR-ELISA is more costly than streptavidin-coated magnetic beads. Although allele-specific hybridization using streptavidin-coated magnetic beads is somewhat costlier than dot-blot hybridization, streptavidin-coated magnetic beads can be reused by washing with alkaline solution. Magnetic bead hybridization is suitable for analysis of a small number of plants with a large number of SNP markers, while dot-blot-SNP is better than magnetic bead hybridization for analysis of a large number of plants with a small number of SNP markers.

In the present study, we repeated analyses to show reliable quantitative data. However, in practical genotyping, it is not necessary to measure absorbance, and genotyping data can be obtained by visual scoring with neither a microplate reader nor repetition. This method is simple and reliable among the methods for allele-specific hybridization, and requires no special equipment except a PCR machine. It will be useful for developing a kit for species identification or genotyping of plants.

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**Supplementary Data**
**Supplementary Table 1**. Sequences of species-specific ITS1 probes and hybridization conditions

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Supplementary Table 1. Sequences of species-specific ITS1 probes and hybridization conditions

| Species name | Accession number of the rDNA sequence | Probe name | Probe sequence(5'→3') | Magnetic bead hybridization condition | Dot-blot hybridization condition | |
|---|---|---|---|---|---|---|
| | | | | Temperature | Temperature | x SSC |
| *Brassica rapa* | AF531563 | BrITS1 | CCTTGGCCAAGACTTCAGTTTT | 50 | 60 | 0.5 |
| *Brassica oleracea* | GQ891879 | BoITS1 | TCACCGGCCCAGTTTCGG | 50 | 60 | 1 |
| *Brassica nigra* | GQ268059 | BniITS1 | TTCAAGACTTACTTAGGTCTCGG | 50 | 60 | 1 |
| *Brassica tournefortii* | AY722428 | BtITS1 | CGTCCCCGATCAAGACT | 50 | 50 | 0.5 |
| *Diplotaxis muralis* | DQ983972 | DmITS1 | TCCTCAGCCAAGTTTATCTTGG | 50 | 50 | 0.5 |
| *Eruca sativa* | DQ249821 | EsITS1 | CGGCCAAGATTTTTGTCTTGGTTG | 50 | 50 | 0.1 |
| *Moricandia arvensis* | DQ249832 | MaITS1 | CCGAGACTTTTGGTCTCGGTTG | 50 | 60 | 1 |
| *Sinapis alba* | AY722487 | SalITS1 | TATGCGTTAAGTTCCCAG | 50 | 40 | 0.5 |
| *Sinapis arvensis* | AY722480 | SarITS1 | AAGACTTCTGTCTCTCGGTCGG | 50 | 50 | 0.2 |
| *Raphanus sativus* | AY722486 | RsITS1 | GTCAAGGCTGGGTCGT | 50 | 60 | 0.5 |

Supplementary Table 2. Sequences of *SRK* allele-specific oligonucleotide probes and hybridization conditions

| *S* haplotypes | Accession number of the *SRK* sequence | Probe name | Probe sequence (5'→3') | Magnetic bead hybridization condition | Dot-blot hybridization condition | | *S* haplotype of interspecific pair |
|---|---|---|---|---|---|---|---|
| | | | | Temperature | Temperature | x SSC | |
| *BrS-12* | AB035503 | BrS-12HV1 | AAACGGCTCACCAGGTC | 50 | 40 | 0.5 | *BoS-51* |
| *BrS-25* | AB370002 | BrS-25HV1 | TATTGAAGACATTCCAGTACATCGG | 50 | 40 | 0.5 | *BoS-14* |
| *BrS-27* | AB089510 | BrS-27HV1 | ATGGAATGAGGACTTTCCAATGC | 50 | 40 | 0.2 | *BoS-8* |
| *BrS-36* | AB039759 | BrS-36HV1 | ATAAAGGCAACTTTCGA | 50 | 40 | 0.5 | *BoS-24* |
| *BrS-41* | AB039762 | BrS-41HV1 | ATCTATCGAGTCATGGAATTTTTCG | 50 | 40 | 0.2 | *BoS-64* |
| *BrS-52* | AB035505 | BrS-52HV1 | TATATATTTAGCGACGACTTTCG | 50 | 40 | 0.1 | - |
| *BrS-54* | AB219161 | BrS-54HV1 | TACTGAAAAGTGGCTTCCAAG | 50 | 40 | 0.5 | *BoS-28* |

*BrS* and *BoS* indicate *S* haplotypes of *B. rapa* and *B. oleracea*, respectively.

Supplementary Table 3. Sequences of primer pairs and oligonucleotide probes of SNP markers and hybridization conditions

| Marker name | Forward Primer (5'→3') | Reverse Primer (5'→3') | Probe | | Magnetic bead hybridization condition | Dot-blot hybridization condition | |
|---|---|---|---|---|---|---|---|
| | | | Allele | Probe sequence(5'→3') | Temperature | Temperature | x SSC |
| BrFLC1 | GCCAATCGGATCGAAACTTA | CAAGAACAACCATGCGCTTA | Wild | TTTAGTTACCTGGCTAG | 40 | 50 | 1 |
| | | | Mutant | TTTAGTTATCTGGCTAG | 40 | 50 | 1 |
| BrFLC2 | TTTCCAATCTGCTGACCAAA | GCAACGAGGCATGTCATCTA | Wild | ACTTCTTATACATGAGA | 40 | 40 | 0.5 |
| | | | Mutant | ACTTCTTAGACATGAGA | 40 | 40 | 0.5 |
| BrGL1 | TGTGTCAAATCCGCTTTTTG | TCTCCCAAATCCAGATGGTC | Wild | CGGTGGATAGTTTTCTT | 40 | 50 | 1 |
| | | | Mutant | CGGTGGATCGTTTTCTT | 40 | 50 | 1 |
| BrMLPK | TTGCTGTTACAGGAGGTTCC | GTATATGACTTGCGTC | Wild | GTGCAAAAGGTCTAGAT | 40 | 60 | 1 |
| | | | Mutant | GTGCAAAACGTCTAGAT | 40 | 50 | 0.5 |
| RsCL0886 | CAGCAGCAGTAGCGTTATGGAT | CGATGATCGTGTCGGAGAACTA | Aokubi | GAGAAAATTAGCCGCAG | 40 | 40 | 1 |
| | | | Saya | GAGAAAATCAGCCGCAG | 40 | 50 | 0.5 |
| RsCL0890 | CGCTCAGAAACTCTGTCGATTC | TCTGCTACCAAACGGGAAGATT | Aokubi | GATCATCATACTCCGACT | 40 | 50 | 1 |
| | | | Saya | GATCATCACACTCCGACT | 40 | 50 | 1 |
| RsCL0976 | CTTTAGCCAGCTTCCCATTTTC | CCTTCACGCTCCTCATTCTCTT | Aokubi | TTATTCTCTAATTGATG | 40 | 40 | 1 |
| | | | Saya | ATTATTCTTTATTGATGC | 40 | 35 | 1 |
| RsCL1019 | TGTTCTTCTCGCCATAAACCAC | CACTCACATTCGATCGGGAATA | Aokubi | TCACTATAAAACCTTCG | 40 | 40 | 1 |
| | | | Saya | TCACTATAGAACCTTCG | 40 | 40 | 1 |
| RsCL1033 | TTGTCATCTCGAAGCCTGATGT | TACAAAGAGGACCAACGGGAAG | Aokubi | ACATAGGTTGAGTTGC | 40 | 40 | 1 |
| | | | Saya | ACATAGGTCGAGTTGC | 40 | 40 | 1 |
| RsCL1062 | CTGGACAATGTAGGGATGTGGA | CGCCAACAAGGCATATGATAGA | Aokubi | CTGATTCGTTCTCGAGG | 40 | 40 | 1 |
| | | | Saya | GATTCGCTTTCTCGACG | 40 | 35 | 1 |
| RsCL1111 | TTGCACACAGGTGATCAAACAG | GTCATGGTAACCAACCAGCATC | Aokubi | TCAAGAGTTCGAACA | 40 | 40 | 1 |
| | | | Saya | TCAAGAGTCTGAACA | 40 | 40 | 0.5 |
| RsCL1112 | TTGCTCTTCGTCTACGATTCCA | GGCAAACTGATTGAACTTGTGC | Aokubi | GTCCTTCAATAATAC | 40 | 35 | 1 |
| | | | Saya | GTCCTTCTATGATAC | 40 | 40 | 1 |
| RsCL1179 | GTAGTGAATGATGGCAGCGTTT | CTTCTTGCTGCTATGCGCTTTA | Aokubi | ATGAAACAACTTCTA | 40 | 40 | 1 |
| | | | Saya | CATGAAAATCGTCTA | 40 | 40 | 1 |
| RsCL1259 | ACTGCGATCTTGAACAATACCC | GCCACGTGTTACTTGTTCGGTA | Aokubi | GAGTCACAGTGGAA | 40 | 40 | 1 |
| | | | Saya | AAGAGTCAGTGGAAG | 40 | 40 | 1 |
| KBrB006C05 | AGTCTAGTGGTCTCTGCAGA | CATTAGCCGCTGCCATCTTA | shogoin | AATTACTTAGAGAATT | 40 | 35 | 1 |
| | | | chiifu | AATTACCTTGAGAATT | 40 | 35 | 1 |
| KBrH013B19 | ACCGAAACTGAAGCCCTGTT | CACTTGCTTCAGAACGCAGA | shogoin | AATCCTAGGATCAGGG | 40 | 40 | 1 |
| | | | chiifu | AATCCTCGGGTCAGGG | 40 | 40 | 1 |
| KBrH014M07 | TGTCTATTACAGCCTGCACC | GATCATCGACCAGTGCAGAA | shogoin | GTGTACTGAGTGTAGGC | 40 | 40 | 1 |
| | | | chiifu | GTGTACTGCGTGTAGGC | 40 | 40 | 0.5 |
| KBrH098A19 | GAATAGCACTCTGCAACTCC | ACCACCGTCACAGCTTTCAA | shogoin | CGGAGTGAGTCGGAGAT | 40 | 50 | 1 |
| | | | chiifu | CGGAGTGACTCGGAGAT | 40 | 40 | 0.5 |
| KBrB068B07 | ACCACAATCGTCGATCGAGA | AAACTCAGCTTCCTCCCAGA | shogoin | CTAGGGCCGAGGCCCGA | 40 | 35 | 1 |
| | | | chiifu | CTAGGGCCCAGGCCCGA | 40 | 50 | 1 |
| BrARC1 | CGCTACTGCGCTCACTCCTCCAAGC | CGTTCTGTACAAGAATCGATAACG | shogoin | CCTGTTGATAGTCTCTG | 40 | 40 | 1 |
| | | | chiifu | CCTGTTGAGAGTCTCTG | 40 | 40 | 1 |
| BrRSCL3279 | TAAGCGTTCACGACTCATCCAT | GGTTCTCCTGAGCGTCAAAAAG | shogoin | TTGGTCAAAACTTATAT | 40 | 35 | 0.5 |
| | | | chiifu | TTGGTCAACACTTATAT | 40 | 40 | 0.5 |
| BrRSCL4309 | CATCACAAGATTCCTCCCTCAA | GATACCATCTACCGTTCGCACA | shogoin | CCCACAACAACAACAAC | 40 | 40 | 1 |
| | | | chiifu | CCCACAACCACAACAAC | 40 | 40 | 1 |
| BrRSCL6068 | GGAAAACCAAAGTGGAGCAA | TGATTCTGACCCTCCAGCTT | shogoin | AAGGAAGCTAGAAGAAA | 40 | 40 | 1 |
| | | | chiifu | AAGGAAGCAAGAAGAAA | 40 | 40 | 1 |
| BrRSCL7471 | GGACATCTCTTCTCGCCTCTTT | CTGTGCATAAGCCATTGAGTCC | shogoin | TGTTAAGGAAAAGTTGA | 40 | 40 | 1 |
| | | | chiifu | TGTTAAGGGAAAGTTGA | 40 | 40 | 1 |

Supplementary Table 4. SNP analysis of cultivars in *B. rapa* by magnetic bead hybridization

| Cultivar | Marker name and allele | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | KBrB006C05s | | KBrH013B19s | | KBrH014M07s | | KBrH098A19s | | KBrB068B07s | | BrRSCL4309 | | BrRSCL6068 | | BrRSCL7471 | |
| | Shogoin | Chiifu | Shogoin | Chiifu | Shogoin | Chiifu | Shogoin | Chiifu | Shogoin | Chiifu | Shogoin | Chiifu | Shogoin | Chiifu | Shogoin | Chiifu |
| Shogoin | 1.00* | 0.00 | 1.00* | 0.06 | 1.00* | 0.23 ±0.25 | 1.00* | 0.00 | 1.00* | 0.70 ±0.13* | 1.00* | 0.28 ±0.20 | 1.00* | 0.63 ±0.18* | 1.00* | 0.08 |
| Chiifu | 0.00 | 1.00* | 0.00 | 1.00* | 0.27 ±0.20 | 1.00* | 0.20 | 1.00* | 0.10 | 1.00* | 0.13 | 1.00* | 1.48* | 1.00* | 0.09 | 1.00* |
| Harusakari P04 | 0.00 | 1.16* | 0.00 | 1.63* | 0.16 | 1.18* | 0.87* | 0.32 ±0.18 | 0.10 | 0.20 | 0.14 | 1.89* | 1.23* | 0.60±0.19* | 0.05 | 0.68 ±0.15* |
| Osome P11 | 0.00 | 1.15* | 0.89* | 0.00 | 0.10 | 1.73* | 0.20 | 1.72* | 0.83* | 1.30* | 0.11 | 1.60* | 0.96* | 0.73* | 0.15 | 1.20* |
| Yellow Sarson | 0.00 | 1.15* | 0.00 | 0.80* | 0.32 ±0.17 | 3.30* | 0.91* | 0.01 | 0.85* | 0.70±0.20* | 0.12 | 2.05* | 1.20* | 0.15 | 0.12 | 0.88* |
| Nozaki | 0.00 | 1.16* | 0.00 | 2.87* | 0.13 | 1.30* | 0.15 | 0.90* | 0.75 ±0.07* | 0.00 | 0.52 ±0.33* | 0.14 | 1.20* | 0.33 ±0.09* | 0.09 | 1.24* |
| Kyouto No.3 | 0.00 | 0.86* | 0.00 | 3.29* | 0.20 | 1.58* | 0.18 | 1.07* | 0.05 | 0.60 ±0.21* | 0.20 | 1.15* | 1.10* | 0.61 ±0.21* | 0.05 | 0.83* |
| Kagakekyu | 0.00 | 0.73* | 0.00 | 1.96* | 0.10 | 0.20 ±0.22 | 0.14 | 0.99* | 0.00 | 0.00 | 0.13 | 1.41* | 0.77 ±0.03* | 0.02 | 0.05 | 0.69 ±0.22* |

These cultivars are open pollinated cultivars, and therefore heterozygous in some genes.

Relative signal intensities (absorbance of 405 nm) to the full matching (1.00) are shown. Values higher than 0.8 are regarded as positive, while those lower than 0.2 are regarded as negative. For the combinations with relative signal intensities between 0.2 and 0.8, analysis was repeated three times and SE was calculated.

Combinations of cultivars and markers that showed positive signals by dot-blot hybridization are indicated by *.

Combinations of cultivars and markers having another SNP within the probe sequence are underlined.

Supplementary Table 5. SNP analysis of cultivars in *R. sativus* by magnetic bead hybridization

| Cultivar | Marker name and allele | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RsCL0886 | | RsCL0976 | | RsCL1033 | | RsCL1062 | | RsCL1111 | | RsCL1112 | | RsCL1179 | | RsCL1259 | |
| | Aokubi | Saya | Aokubi | Saya | Aokubi | Saya | Aokubi | Saya | Aokubi | Saya | Aokubi | Saya | Aokubi | Saya | Aokubi | Saya |
| Aokubi | 1.00* | 1.91* | 1.00* | 1.00* | 1.00* | 0.04 | 1.00* | 0.01 | 1.00* | 0.28 ±0.17* | 1.00* | 0.00 | 1.00* | 0.00 | 1.00* | 0.51 ±0.18* |
| Saya | 0.00 | 1.00* | 0.26 ±0.09 | 0.02 | 0.15 | 1.00* | 0.02 | 1.00* | 0.01 | 1.00* | 0.12 | 1.00* | 0.00 | 1.00* | 0.13 | 1.00* |
| Shogoin | 0.00 | 1.45* | 1.67* | 0.00 | 0.76 ±0.08* | 0.03 | 0.03 | 0.87* | 1.55* | 0.05 | 0.24 ±0.08 | 1.17* | 0.00 | 0.00 | 0.31 ±0.07 | 1.02* |
| Sakurajima | 0.00 | 1.75* | 0.38 ±0.12* | 0.88* | 0.38 ±0.06* | 1.97* | 0.91* | 0.02 | 1.30* | 0.51±0.23* | 0.70 ±0.13* | 1.01* | 0.00 | 0.00 | 0.12 | 1.11* |
| Moriguchi | 1.21* | 1.73* | 2.30* | 0.00 | 0.80* | 0.08 | 0.92* | 0.02 | 1.19* | 0.09 | 0.14 | 0.60 ±0.20* | 0.00 | 0.73 ±0.13* | 2.09* | 0.15 |
| Karaine | 1.20* | 0.24 | 2.00* | 0.00 | 1.04* | 0.09 | 1.12* | 0.03 | 1.59* | 0.04 | 0.16 | 0.81* | 0.00 | 0.34 ±0.10* | 0.81* | 0.68 ±0.20* |
| Koushin | 0.03 | 2.33* | 0.00 | 1.03* | 0.16 | 1.74* | 0.97* | 0.03 | 1.43* | 0.02 | 0.13 | 1.00* | 0.72 ±0.09* | 0.00 | 0.87* | 0.16 |
| Comet | 0.00 | 2.21* | 0.00 | 1.58* | 0.16 | 0.09 | 0.03 | 0.03 | 0.01 | 0.04 | 0.70 ±0.21* | 0.10 | 0.09 | 0.00 | 0.31 ±0.06 | 1.00* |

These cultivars are open pollinated cultivars, and therefore heterozygous in some genes.

Relative signal intensities (absorbance of 405 nm) to the full matching (1.00) are shown. Values higher than 0.8 are regarded as positive, while those lower than 0.2 are regarded as negative. For the combinations with relative signal intensities between 0.2 and 0.8, analysis was repeated three times and SE was calculated.

Combinations of cultivars and markers that showed positive signals by dot-blot hybridization are indicated by *.

Combinations of cultivars and markers having another SNP within the probe sequence are underlined.